Cloning, Characterization, Controlled Overexpression, and Inactivation of the Major Tributyrin Esterase Gene of Lactococcus lactis

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The gene encoding the major intracellular tributyrin esterase of Lactococcus lactis was cloned using degenerate DNA probes based on 19 known N-terminal amino acid residues of the purified enzyme. The gene, named estA, was sequenced and found to encode a protein of 258 amino acid residues. The transcription start site was mapped 233 nucleotides upstream of the start codon, and a canonical promoter sequence was identified. The deduced amino acid sequence of the estA product contained the typical GXSXG motif found in most lipases and esterases. The protein was overproduced up to 170-fold in L. lactis by use of the nisin-controlled expression system recently developed for lactic acid bacteria. The estA gene was inactivated by chromosomal integration of a temperature-sensitive integration vector. This resulted in the complete loss of esterase activity, which could then be recovered after complementation of the constructed esterase-deficient strain with the wild-type estA gene. This confirms that EstA is the main enzyme responsible for esterase activity in L. lactis. Purified recombinant enzyme showed a preference for short-chain acyl esters, surprisingly also including phospholipids. Medium- and long-acyl-chain lipids were also hydrolyzed, albeit less efficiently. Intermediate characteristics between esterases and lipases make intracellular lactococcal EstA difficult to classify in either of these two groups of esterolytic enzymes. We suggest that, in vivo, EstA could be involved in (phospho)lipid metabolism or cellular detoxification or both, as its sequence showed significant similarity to S-formylglutathione hydrolase (FGH) of Paracoccus denitrificans and human EstD (or FGH), which are part of a universal formaldehyde detoxification pathway.

A large number of microbial lipolytic enzymes have been identified and characterized to date. These lipolytic enzymes, mainly esterases and lipases, belong to the general class of carboxylic ester hydrolases (EC 3.1.1) but differ in substrate specificity and type of enzyme kinetics (50). Applications of microbial lipolytic enzymes are widely found in the food, detergent, pharmaceutical, and chemical industries (19). The breakdown of milk fat by lipases and esterases is one of the main biochemical events that occur during cheese ripening, and it contributes to flavor development. The contribution of the native milk lipase to lipolysis in cheese is significant only in those varieties produced with raw milk, as this enzyme is activated during heat treatment. In soft blue-veined cheeses, the extracellular lipase from the mold Penicillium roquefortii contributes to the intense and characteristic flavor (26). In other varieties, like cheddar cheese, only a low level of milk fat hydrolysis occurs due to the weak lipolytic and esterolytic activities of the starter bacteria (7, 37). However, the small amounts of short-chain fatty acids are important because they have a low sensorial detection threshold (36) and they contribute to flavor balance (7).

Reports have been published on the purification and partial characterization of intracellular esterases of lactococci (5, 24, 48). The Lactococcus lactis ACA-DC 127 esterase, a monomeric 68-kDa enzyme, belongs to the class of serine esterases and hydrolyzes synthetic substrates (p-nitrophenyl [p-NP] esters shorter than or equal to C_8) (48). The recently purified esterases of L. lactis NCDO 763 and E8 share several characteristics: they are intracellular, the estimated molecular mass of the monomer is 29 kDa, the optimal activity is at pH 7.0 to 8.0, and the reported N-terminal sequences are identical (5, 24). The enzyme from L. lactis NCDO 763 showed higher activities with p-NP-butanoyl, like the esterase of ACA-DC 127, but it also hydrolyzed p-NP esters longer than C_8 (5). On the other hand, the lactococcal enzyme described by Holland and Coolbear (24) had p-NP-butanoyl, tributyrin, and milk fat hydrolyase activity but was unable to hydrolyze triacylglycerols with long-chain fatty acids such as tripalmitin or triolein. To our knowledge, genetic characterization of the lipolytic enzymes produced by Lactococcus has not been reported up to now, in spite of the great developments in genetic engineering of the industrially important lactic acid bacteria in the past years.

This paper describes the cloning and characterization of the gene encoding the major tributyrin esterase of L. lactis subsp. lactis B1014, which is very similar to the E8 esterase (R. Holland and T. Coolbear, unpublished data). Further work was also carried out with Lactococcus lactis subsp. cremoris MG1363, a plasmid-free model strain of Lactococcus. Several L. lactis strains that overproduced either B1014 or MG1363 esterase were constructed by using the recently developed nisin-controlled expression system (12, 29, 30). The substrate selectivity

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of the esterases purified from the overproducing strains was
defined by using p-NP-acyl ester substrates.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacteria and plasmids used or constructed for this study are listed in Table 1. Unless otherwise indicated, L. lactis strains were routinely grown without aeration at 30°C in M17 (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% (wt/vol) glucose. Escherichia coli MC1061 (3) was used as a host for cloning experiments, and it was grown with aeration in L broth-based medium at 37°C (42). When antibiotics were added, the final concentrations used were as follows: ampicillin, 50 μg ml⁻¹; chloramphenicol, 10 μg ml⁻¹ for E. coli and 7.5 μg ml⁻¹ for L. lactis; tetracycline, 10 μg ml⁻¹ for E. coli and 7.5 μg ml⁻¹ for L. lactis.

The plasmids vectors used in the cloning experiments with E. coli were pUC18 and pUC19 (54) and pGEM-SZ(+) (Promega Corp.). In L. lactis, the plasmids pNZ8020 and pNZ8030 (12, 28, 30) and pGhost8 (35) were used. pGhost8 has a temperature-sensitive origin of replication; its replication is normally blocked at temperatures higher than 37°C.

DNA isolation and manipulation. Isolation of plasmid DNA from E. coli and transformations of E. coli strains were carried out according to established procedures (42). Both plasmid and chromosomal DNAs of L. lactis were isolated as described previously (52). L. lactis cells were transformed by electroporation (53). DNA fragments were isolated from agarose gels by using the GlassMAX DNA isolation matrix system (BRL Life Technologies, Inc., Gaithersburg, Md.). Restriction enzymes, T4 DNA ligase, and other DNA-modifying enzymes were purchased from GIBCO/BRL Life Technologies, New England Biolabs Inc., or Promega Corp. and used as recommended by the manufacturer. Oligonucleotides were purchased from Pharmacia. Cloning procedures, agarose gel electrophoresis, radiolabeling of oligonucleotides, and Southern blot hybridizations were performed as described previously (27).

Cloning of the tributyrin esterase gene. Chromosomal DNA from L. lactis B1014 was isolated and digested with several restriction enzymes. The B1014 tributyrin esterase gene was localized by using the degenerate oligonucleotides 5'-GCNGTNTAT(CT)AA(CT)AT(CT)GA(AG)TA(CT)TA-3' and 5'-TT(AG)ITNAC(TT)TT(CT)C(GT)(AG)TTCATNC3', which were based on parts of the first 19 N-terminal amino acids, i.e., AVINJEY and GMRNKVYN, respectively, of the tributyrin esterase from L. lactis E8 (24). Southern hybridization revealed a 4.5-kb EcoRI-SorI fragment from chromosomal DNA that hybridized with both oligonucleotides, and fragments of approximately this size were cloned into the EcoRI and SorI restriction sites of pUC18. Colony blot hybridization localized a clone that contained the 4.5-kb EcoRI-SorI hybridizing fragment, which was named pNZ9301. Restriction analysis of the insert and Southern hybridization demonstrated that the tributyrin esterase gene was close to the EcoRI site of the fragment. Sequence analysis showed that the insert contained the 5' end of the tributyrin esterase gene, but the whole sequence was not present in this fragment. The determined sequence of the cloned EcoRI-SorI fragment revealed an Asel restriction site close to the EcoRI site. A 1.7-kb Asel-HindIII fragment of chromosomal DNA from B1014 was subsequently isolated after hybridization with the LipE probe (5'-CCCAACAGATATTCCGTTG-3'). This oligonucleotide was designed after the nucleotide sequence of the tributyrin esterase gene close to the EcoRI site that had been determined (nucleotides [nt] 392 to 411) (Fig. 1). This 1.7-kb Asel-HindIII fragment was cloned into the HindII-HindIII sites of pUC19, resulting in plasmid pNZ9302. Together, the two DNA chromosomal fragments present in pNZ9301 and pNZ9302 contained the complete open reading frame (ORF) encoding the B1014 tributyrin esterase gene (estA).

The estA gene from L. lactis MG1363 was amplified by PCR with oligonucleotides LipPCR1 (5'-GACTTCCACAGCGCACTCTTG-3') and Lip8 (5'-ATA CCAGACGGACACAA-3'), designed on the basis of the nucleotide sequence of cloned chromosomal fragments from B1014, using the Advantage genomic polymerase mix (Clontech Laboratories, Inc., Palo Alto, Calif.). The amplified 1.6-kb PCR product, which contained the complete MG1363 esterase gene, was cloned in pGEM-SZ(+) using the pGEM-T Vector System (Promega Corp.), resulting in pNZ9333.

DNA sequencing and nucleotide and deduced protein sequence analysis. DNA sequence analysis was performed by the dideoxy chain termination procedure (43) with fluorescein-labeled primers and an AutoRead sequencing kit (Pharmacia). The nucleotide sequence was determined in both orientations with an ALF automatic DNA sequencer (Pharmacia Biotech). Computer analysis of DNA sequences and the deduced amino acid sequences was performed with the PC/GENE program, version 6.70 (IntelliGenetics). Database searching for related proteins was performed with BLAST (1), TFASTA (39), and

<table>
<thead>
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<th>Strain or plasmid</th>
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<td>New Zealand Dairy Research Institute culture collection</td>
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- a Ap', Cm', Te', resistance to ampicillin, chloramphenicol, and tetracycline, respectively.
- b Promega Corp. technical bulletin no. 150.
similar strategy was used to construct pNZ9331, which contains the MG1363 digested with Nco, containing an estA gene in pNZ8020 digested with PstI and cloned into pNZ8030. The missing part of the estA promoter was cloned in pNZ8020 digested with Smal, yielding pNZ9308. Similarly, pNZ9330 was obtained by cloning an 856-bp EcoRI-SphI fragment carrying the MG1363 estA gene in pNZ8020 digested with EcoRI and XhoI (Klenow fragment). The primers used for cloning the B1014 estA gene in pNZ8030 were Eco5′GTCGCCATCTGAGGAATTCTCGTTGCTTG-3′ and pNZ9331 was constructed previously was used to deliver the wild-type MG1363 estA gene, and EstB4 (complementary to the chromosomal sequence downstream of the MG1363 estA gene; positions 1364 to 1385 in Fig. 1), complementing the estA gene-deficient derivative. Plasmid pNZ9332 was constructed by transforming pNZ9331 constructed previously with the oligonucleotide with the sequence 5′GGCTCTTCGTTGGTTTACACCCAGTTTGTAATTCCAGGA-3′ (underlined), and LipN1 (5′-CTACTACTCACTCACTGCTATTGC-3′) was used as a primer.

**FIG. 1.** Nucleotide sequence of the L. lactis B1014 tributyrin esterase gene. The predicted amino acid sequence is given below the nucleotide sequence in the standard one-letter code. The stop codon is marked by an asterisk. The transcription initiation site mapped by primer extension is marked by a vertical arrow (Fig. 2). The nucleotide sequence reported in this paper have been submitted to GenBank and assigned accession numbers AY435378 to AY435383.

**TABLE 2.** Multiple sequence alignment of the B1014 estA promoter as described previously (28). The oligonucleotide with the sequence 5′-GTGGGATCCCTGAGTTCTTCAACTCTGCTATTGC-3′, which is complementary to positions 119 to 123, was used as a primer.

**Enzyme purification.** Enzyme purification was carried out by the method described for the purification of tributyrin esterase (22) with the following modifications. The enzyme was purified to homogeneity from the complemented derivative (see Materials and Methods, “Purification of tributyrin esterase from L. lactis and gel filtration (Sephadex G150 Superfine, 90- by 1.6-cm column; Pharmacia).”)

**Results**

Cloning of the tributyrin esterase gene, estA. Two oligonucleotides based on the reported N-terminal sequence of the tributyrin esterase from L. lactis E8 (24) were used to screen for the tributyrin esterase gene from L. lactis B1014. Two chromosomal DNA fragments, together encoding the putative tributyrin esterase, were cloned (see Materials and Methods, “Purification of tributyrin esterase from L. lactis and gel filtration (Sephadex G150 Superfine, 90- by 1.6-cm column; Pharmacia).”)

**Protein determinations.** Protein quantities were estimated by the method of Bradford (2) with a Bio-Rad kit and bovine serum albumin as the protein standard.

**Protein acid sequence.** The terminal sequence of B1014 EstA was determined at the Protein Sequencing facility in Delft (The Netherlands).

**Nucleotide sequence accession numbers.** The nucleotide sequences reported in this paper have been submitted to GenBank and assigned accession numbers AF157484 and AF157681 for Lactococcus lactis subsp. lactis B1014 and L. lactis subsp. cremoris MG1363, respectively.
and the corresponding gene was named estA. The \textit{L. lactis} MG1363 \textit{estA} gene was cloned by using two primers which were based on the nucleotide sequence surrounding the B1014 tributyrin esterase gene.

**Nucleotide sequence of \textit{L. lactis estA}.** The complete nucleotide and the deduced amino acid sequences of the \textit{estA} gene of \textit{L. lactis} B1014 obtained by PCR were identical to that of the two initially cloned fragments and are shown in Fig. 1. One complete 774-bp ORF (ATG initiation codon at nt 295 and TAA termination codon at nt 1072) was identified in the sequence. This ORF could encode a polypeptide of 258 amino acids with a deduced molecular weight of 29,640. A good potential Shine-Dalgarno sequence, 5’-AAGGAG (AGG [25°C] = −12.8 kcal [1 kcal = 4.184 kJ/mol]) (47), which is complementary to the 3’ end of the \textit{L. lactis} 16S rRNA (4), was found 13 bases upstream from the initiation codon ATG. The transcription initiation site was determined by primer extension analysis of the upstream region of the B1014 \textit{estA} gene and was localized 233 nt upstream from the ATG start codon at a guanine residue (nt 62) (data not shown). This transcription start site is preceded by the canonical −35 and −10 promoter sequences at positions 25 to 30 and 50 to 55, respectively, that are spaced by 20 nt. The putative −10 region is immediately preceded by the sequence TGN, which is often found in lactococcal promoters (14). The sequence downstream of the stop codon lacks any inverted repeats. No additional ORF was found between the promoter and the transcription start site of B1014 \textit{estA}. Analysis of the ORF indicated that the protein lacks a classical secretion signal sequence at the N terminus of the esterase. The amino acid sequence GLSMG, starting at residue 649, fits the GXSXG motif found in most bacterial and eukaryotic serine hydrolases, like lipases and esterases (8). The sequencing of the PCR-amplified product from MG1363 chromosomal DNA cloned in pNZ9333 revealed an ORF of the same size as and with 84% similarity to B1014 \textit{estA}, preceded by an identical putative Shine-Dalgarno sequence. The deduced product of MG1363 \textit{estA} also contained the typical GXSXG motif.

**Comparison of amino acid sequences.** Database searches revealed several (putative) proteins with 30 to 35% sequence identities to EstA from \textit{L. lactis} B1014. These homologous proteins could be divided into three groups, as summarized in Table 2. The highest similarity (group A) was with XynC, an acetyl esterase of similar size (266 residues) from the thermophile \textit{Caldocellum saccharolyticum} (34), and also with the N-terminal domain of XynZ, a much larger protein of 837 residues from the thermophile \textit{Clostridium thermocellum} (20, 21). Group B contains many related proteins from various \textit{Myco-} bacteria species (of which only a few are listed in Table 2) and from \textit{Corynebacterium glutamicum}, all of which are listed as extracellular α-antigens with a possible function in fibronectin binding (10, 25, 38, 40, 46). Group C contains the human esterase EstD (31, 55), which is identical to S-formylglutathione hydrolase (FGH) (15); this group also includes the FGHs of \textit{Paracoccus denitrificans}, \textit{Anabaena azollae}, and \textit{Synechocystis} \textit{cerevisiae} (9, 23, 44) and several highly related putative proteins that have recently been discovered through genome sequencing of \textit{Arabidopsis thaliana}, \textit{Synechocystis}, \textit{Haemophilus influenzae}, and \textit{Escherichia coli}. All of the related group C enzymes are presumed intracellular, since they do not contain a signal sequence, and they are all similar in size (266 to 299 residues) to \textit{L. lactis} B1014 \textit{EstA}.

A multiple sequence alignment of the most homologous regions of these (putative) proteins is shown in Fig. 2. In the central section, Ser121 of \textit{L. lactis} B1014 is in the consensus sequence GXSXG around the catalytic serine typical of all lipases and esterases. Near the C terminus, a conserved His231 is found that is predicted to be the catalytic histidine (8). The catalytic Asp/Glu is more difficult to identify, since the surrounding sequence in lipases and esterases is not highly conserved (8). However, the alignment suggests that Asp202 may be the catalytic residue, since this residue is conserved in groups A and C but not in group B of binding proteins. The sequence around residues His44 and Gly45 of B1014 esterase closely resembles the oxyanion hole region of lipases (11). The deduced amino acid sequence of the product of the MG1363 \textit{estA} gene is very similar (less than 7% difference) to EstA from B1014 (data not shown).

**Overproduction of esterase in \textit{L. lactis}.** The estA coding sequences of B1014 and MG1363 were cloned in two expression vectors under the control of the \textit{nisA} promoter. This expression system allows the transcription of the cloned genes to be activated after induction with subinhibitory amounts of nisin A (29). Plasmids pNZ9308 and pNZ9330 are derivatives of pNZ8020 and carry the \textit{estA} gene from B1014 and MG1363, respectively, translationally fused to the \textit{nisA} promoter. These
constructs were introduced into *L. lactis* NZ9000 and NZ9800. Cell extracts of the transformants were used to determine the esterase activity with *p*-NP-butanoate as the substrate after induction with 3 ng of nisin A ml\(^{-1}\) and in the absence of nisin A. Cultures of the wild-type strains were used as controls; the specific esterase activities were 0.06 \(\mu\)mol mg\(^{-1}\) min\(^{-1}\) for B1014 esterase and 0.10 \(\mu\)mol mg\(^{-1}\) min\(^{-1}\) for MG1363 esterase (Fig. 3). The esterase activity in strain NZ9000 harboring pNZ9308, which contains the *estA* gene from B1014, was about 50 times higher after nisin induction than in the uninduced control, which had a level of activity comparable to the wild-type strain. The overproduction of EstA in NZ9000 with pNZ9330, which contains the *estA* gene from MG1363, was even higher, accounting for about a 170-fold increase. When these plasmids were introduced in strain NZ9800, the esterase activity also increased after induction with nisin, but only 28-

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**FIG. 2.** Multiple-sequence alignment of (putative) proteins with sequence homology to EstA of *L. lactis* B1014 (LL EstA), *Caldocellum saccharolyticum* acetyl esterase XynC (CS XynC), *Clostridium thermocellum* XynZ (CT XynZ), *Mycobacterium scrofulaceum* \(\alpha\)-antigen (MS \(\alpha\)-a), *Mycobacterium avium* antigen 85B (MA A85B), *Mycobacterium leprae* antigen 85C (ML A85C), *Corynebacterium glutamicum* PS1 protein (CG PS1), human esterase D (HS EstD), *Anabaena azollae* FGH (AA FGH), *Arabidopsis thaliana* T32G6.5 hypothetical gene product (AT T32G6.5), *Saccharomyces cerevisiae* FGH (SC YJL068c), *P. denitrificans* FGH (PD FGH), *H. influenzae* 0184 hypothetical gene product (HI 0184), *Synechocystis* sp. hypothetical gene product (SS ?), and *E. coli* YeiG and YaiM hypothetical gene products (EC YeiG and EC YaiM). Only relevant regions of highest homology are shown. The numbering at the top is that of EstA, while numbers before and after each sequence indicate the numbers of additional N-terminal and C-terminal residues, respectively. The consensus sequence shows fully conserved residues in uppercase and highly conserved residues in lowercase letters. The putative catalytic Ser, Asp, and His residues are indicated in boldface and by asterisks.

**FIG. 3.** Expression levels of *estA* in different *L. lactis* strains and constructs. B1014 and MG1363 are wild-type tributyrin esterase producer strains, NZ9000 and NZ9800 are nisin-sensitive strains for induction of gene expression, and NZ9340 is an esterase-deficient mutant. Plasmids pNZ9308 (B1014 *estA*) and pNZ9330 (MG1363 *estA*) are derivatives of pNZ8020 (transcriptional fusion vector), while pNZ9310 (B1014 *estA*) and pNZ9331 (MG1363 *estA*) are derivatives of pNZ8030 (translational fusion vector). The esterase activity was determined in cell extracts after induction with 3 ng of nisin A ml\(^{-1}\) and in the absence of nisin A, and it is expressed as micromoles of *p*-nitrophenol liberated from *p*-NP-butanoate per minute per milligram of protein.
fold for pNZ9308 and 50-fold for pNZ9330. Overproduction of EstA was found as well in NZ9000 and NZ9800 carrying these est genes translationally fused to the start codon of nisA. Analysis of the intracellular protein of all these constructs on sodium dodecyl sulfate-PAGE gels revealed overproduction of the 29-kDa tributyrin esterase protein after induction with nisin A (data not shown). Esterase activity was also assayed with p-NP-butanoate and tributyrin as substrates in whole cells, cell homogenates, cell-free supernatants, and cell-free pellet fractions prepared from both NZ9800 containing pNZ9308 (estA from B1014) and B1014. Esterase activity in the cell homogenate was 80- to 100-fold higher in strain NZ9800 containing pNZ9308 than in wild-type B1014 when expressed as specific activity based upon the amount of protein (data not shown).

Disruption of estA. The chromosomal estA gene of NZ9000 was inactivated by gene disruption with a temperature-sensitive integration vector. This vector can be introduced and was inactivated by gene disruption with a temperature-sensitive integration vector. This vector can be introduced and was inactivated by gene disruption with a temperature-sensitive integration vector. This vector can be introduced and was inactivated by gene disruption with a temperature-sensitive integration vector. This vector can be introduced and was inactivated by gene disruption with a temperature-sensitive integration vector.

The substrate specificity of purified EstA was determined by use of p-NP esters of fatty acids ranging in chain length from C₁₂ (ethanoate) to C₁₆ (hexadecanoate). Highest activity was observed with p-NP-hexanoate (C₆). Km and V_max values were calculated from Lineweaver-Burk plots using a least-squares best fit of the Michaelis-Menten equation (Table 3). The calculated V_max values were substantially higher than the observed maximum rates of reaction, particularly with p-NP esters of fatty acids of longer chain length. EstA was not active on p-NP ester substrates with an acyl chain length greater than C₁₂ (dodecanoate). The triacylglycerol tributyrin was also a hydrolytic substrate for EstA from B1014. The maximum specific activity measured with tributyrin as the substrate was 8 µmol of butyrate released per min per mg of enzyme protein. Moreover, activity was assayed with the phospholipid analogs diC₆-dithioPC and diC₈-dithioPC (Fig. 4). The enzyme showed relatively good phospholipase activity on the monomeric diC₆-dithioPC, which increased gradually with the

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</table>

*K_m and V_max were calculated from Lineweaver-Burk plots of enzyme activity on p-NP ester substrates by using a least-squares best fit of the Michaelis-Menten equation. —— no enzyme activity.

Purification and substrate specificity of EstA from B1014. The overproducing strain NZ9800 containing pNZ9308, which carries the estA gene from B1014 translationally fused to the nisA promoter, was used as the source of tributyrin esterase. Enzyme purification was monitored by the assay of p-NP-butanoate and tributyrin esterase activities at each step. From an initial 500 mg of protein in a cell homogenate prepared from a 4-liter culture of strain NZ9800 containing pNZ9308, the final yield was 8 mg of essentially pure tributyrin esterase protein. The first 20 amino acid residues of the purified B1014 EstA were identical to the N-terminal sequence of the enzyme purified from L. lactis E8 (24). B1014 tributyrin esterase purified from the overproducing strain behaved in the same manner during purification and PAGE under either denaturing or non-denaturing conditions as the enzyme isolated from strain E8.

To further confirm this result, we disrupted the estA gene from B1014 translationally fused to the nisA promoter, was used as the source of tributyrin esterase. Enzyme purification was monitored by the assay of p-NP-butanoate and tributyrin esterase activities at each step. From an initial 500 mg of protein in a cell homogenate prepared from a 4-liter culture of strain NZ9800 containing pNZ9308, the final yield was 8 mg of essentially pure tributyrin esterase protein. The first 20 amino acid residues of the purified B1014 EstA were identical to the N-terminal sequence of the enzyme purified from L. lactis E8 (24). B1014 tributyrin esterase purified from the overproducing strain behaved in the same manner during purification and PAGE under either denaturing or non-denaturing conditions as the enzyme isolated from strain E8. The substrate specificity of purified EstA was determined by use of p-NP esters of straight-chain fatty acids ranging in chain length from C₁₂ (ethanoate) to C₁₆ (hexadecanoate). Highest activity was observed with p-NP-hexanoate (C₆). Km and V_max values were calculated from Lineweaver-Burk plots using a least-squares best fit of the Michaelis-Menten equation (Table 3). The calculated V_max values were substantially higher than the observed maximum rates of reaction, particularly with p-NP esters of fatty acids of longer chain length. EstA was not active on p-NP ester substrates with an acyl chain length greater than C₁₂ (dodecanoate). The triacylglycerol tributyrin was also a hydrolytic substrate for EstA from B1014. The maximum specific activity measured with tributyrin as the substrate was 8 µmol of butyrate released per min per mg of enzyme protein. Moreover, activity was assayed with the phospholipid analogs diC₆-dithioPC and diC₈-dithioPC (Fig. 4). The enzyme showed relatively good phospholipase activity on the monomeric diC₆-dithioPC, which increased gradually with the
substrate concentration, but no activity was detected on micellar diC12-dithioPC at any of the concentrations assayed (up to 2.4 mM).

DISCUSSION

The estA genes of L. lactis B1014 and MG1363 were cloned via reverse genetics with oligonucleotides designed on the basis of the published amino-terminal sequence of the purified tributyrin esterase from L. lactis E8 (24). The similarity of the estA sequences (84%) from B1014 and MG1363 is slightly higher than that of other genomic sequences from L. lactis subsp. lactis and L. lactis subsp. cremoris, the divergence of which is estimated to be between 20 and 30% (18). It is noteworthy that the N-terminal sequences of the E8, B1014, and MG1363 esterases are very similar to the one reported recently for L. lactis NCDO 763 (5). The predicted size of the estA gene product (29 kDa) is in accordance with that published for the monomers of the purified enzymes from L. lactis E8 and NCDO 763, which are organized as tetramers and trimers, respectively (5, 24). The identity between the deduced N-terminal sequence of EstA from B1014 and MG1363 and that of the enzymes purified from E8 and NCDO 763 (5, 24) shows that the N-terminal part of EstA is not subjected to any modification, except for the removal of the N-terminal methionine. The absence of a classical N-terminal secretion signal sequence in EstA is in agreement with the published intracellular location of the lactococcal esterases (5, 24). The promoter –35 and –10 regions, according to the identified transcription initiation site, conform to the promoter consensus sequence for lacticocci and other gram-positive bacteria, including a TG dinucleoside located 1 bp upstream of the –10 sequence (14). Although rho-independent terminators, such as inverted repeat sequences potentially capable of forming stem-loop structures, are common in L. lactis, no obvious transcription termination structure was found downstream of the TAA stop codon. The absence of a putative stem-loop structure in this part of the sequence could result in a higher turnover of mRNA, which could explain the lack of success in detecting clear estA transcripts (data not shown). It is tempting to speculate that the noncoding region of the estA mRNA (233 bp upstream of the estA start codon) could play a role in the control of gene expression. Nucleotide sequences located between the promoter and the structural gene have been shown to be involved in a variety of regulatory mechanisms for many metabolic genes in gram-positive bacteria (6, 33, 41).

Lactococcal EstA has similarity with various proteins that have been grouped on the basis of their function and/or origin, i.e., xylan/cellulose-degrading enzymes including an acetyl esterase (20, 34), extracellular fibronectin-binding proteins (38, 40), and a group including various intracellular esterases (15, 23, 44) (Table 2). Alignment of these proteins, which showed highest homology to L. lactis B1014 EstA, revealed that the longest conserved sequence contained the typical GXSG box that is found in serine hydrolases such as lipases, esterases, and some proteinases (8). This region has been suggested as the presumptive active site of human EstD, based on homology studies with several esterases (55). Some serine group reagents, such as phenylmethylsulfonfluoride or diisopropylfluorophosphate and Pefabloc, have been shown to inhibit the enzymatic activity of purified esterases of L. lactis ACA-DC 127 and NCDO 763, while inhibitors of metalloenzyme and cysteine enzymes did not have any effect (5, 48). These results, taken together, suggest that Ser121 of L. lactis B1014 EstA is the nucleophilic serine of the active site, which is usually well conserved in many hydrolytic enzymes and forms an acyl intermediate with the substrate. The EstA residues Asp202 and His231 present in conserved sequences located in the carboxyl terminus presumably also participate in catalysis as members of the triad; however, they have not been identified as such for any of the proteins listed in Table 2. The importance of a histidine residue for the esterase activity of L. lactis NCDO 763 has been pointed out by Chich et al. (5), who reported a strong inhibition of this activity by 3,4-dichloroisocoumarin, which binds to the His residue of the catalytic triad of serine enzymes. The residues Ser121, Asp202, and His231 of EstA conform to the typical catalytic triad of lipases and esterases that generally consists of Ser-Asp/Glu-His (8). No disulfide bridge is present in the lactococcal EstA sequence, which contains only a single cysteine residue at position 198, in contrast to several lipases and esterases that contain two or three conserved disulfide bridges important for substrate binding and/or recognition (8). EstA Cys198 forms no part of the active site, but it could be important for the enzymatic activity of EstA. This is supported by the observed inhibition of enzymatic activity in lactococcal esterases by Hg2+2, which reacts with thiol groups (5, 48). A sulphydryl group is also essential for the enzymatic activity of human EstD (32).

The estA genes from both lactococcal strains B1014 and MG1363 were overexpressed efficiently in L. lactis under the control of the nisA promoter with the nisin-controlled expression system (12, 13, 30). Hydrolysis of p-NP-butanoate by these esterase-overproducing strains increased from 28- to 170-fold, respectively, after induction with nisin compared with the uninduced cultures. Higher hydrolysis of tributyrin was also detected in NZ9800 carrying pNZ9308, which contains the estA gene from B1014 transcriptionally fused to the nisA promoter. These results indicate that tributyrin and p-NP-butanoate hydrolysis are catalyzed by the same enzyme. The disruption of the estA gene in MG1363 with an integration vector containing a thermosensitive replicon resulted in the complete loss of esterase activity. This activity was successfully restored when the esterase-deficient mutant (NZ9340) was complemented with a plasmid containing the estA gene from the wild-type strain. Consequently, our results demonstrate that EstA is the major esterase in L. lactis.

The substrate specificity of B1014 esterase was studied by using purified enzyme from the overproducing strain NZ9800 carrying pNZ9308. EstA from B1014 showed the highest activity against low-molecular-weight substrates (p-NP-butanoate and tributyrin). This preference for esters of short-chain fatty acids is characteristic of esterases, and it is shared by other lactococcal esterases (5, 24, 48). Lactococcal EstA hydrolyzed phospholipids with medium-chain fatty acid residues (diC12-dithioPC), and the activity showed a typical hyperbolic dependence on substrate concentration similar to that of pancreateatic phospholipase A1 on monomeric substrates (51). However, no increase in enzyme activity was observed at high diC12-dithioPC concentrations, which will favor micelle formation, or against micellar diC12-dithioPC. This is in contrast to the behavior of pancreatic phospholipase A2, whose catalytic efficiency rises abruptly when similar substrates are in the aggregated form (51). This indicates that lactococcal EstA lacks, at least with the phospholipid substrates assayed, the property of interfacial activation that is also characteristic of lipases.

It can be concluded that EstA does not seem to be an essential enzyme for L. lactis, as no difference in growth was observed between the wild-type and mutant strains in the medium and conditions used in this study, i.e., M17 medium supplemented with glucose and 30 or 37°C. It is difficult to ascertain the physiological role of this lactococcal esterase, but some options may be considered. Esterases from Lactococcus
may be involved in the metabolism or turnover of bacterial lipids, taking into account that the enzyme has the ability to hydrolyze different soluble lipid substrates, including triacylglycerols and phospholipids. Lactic acid bacteria are also able to produce free fatty acids from mono- and diacylglycerides (45). This hypothesis is supported by an early study on lactococcal lipolytic activity that revealed rather intense activity in Lactococcus against its own neutral lipids (49). Interestingly, the clear homology found between the lactococcal esterase and FGH from humans, also known as esterase D (15), and Amidohydrolases, Saccharomyces cerevisiae, and lycase FGHs (9, 23, 44) suggests that this enzyme could play a role in detoxification. FGH is part of a glutathione-dependent formaldehyde detoxification pathway (23). The wide distribution of FGH (yeasts, bacteria, and mammals) has led these authors to suggest that this is a universal detoxification pathway, and it is possible that this pathway also plays a role in Lactococcus.

The availability of lactococcal esterase in high quantities will facilitate further kinetic and structural characterization of the enzyme and a closer definition of its natural lipid substrates. In addition, esterase-overproducing and esterase-deficient strains will allow us to determine the impact of this lactococcal enzyme on flavor formation in dairy products. The assessment of this function has been hampered up to now by the lack of isogenic strains with various esterase activities.

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